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Targeting the anti-apoptotic Bcl-2 members using non-peptide, small-molecule inhibitors is a new and exciting therapeutic strategy. Our work has led to the discovery of potent, non-peptide small-molecule inhibitor apogossypolone that not only binds to Bcl-2 and Bcl-xL proteins but also Mcl-1. Consistent with its strong binding affinity to Bcl-2 members, apogossypolone potently and effectively inhibits cancer cell growth in androgen-independent human prostate cancer PC-3 and DU-145 cell lines. Extensive in vitro and in vivo studies are being carried out to further test its therapeutic potential for the treatment of advanced, androgen-independent human prostate cancer.

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Introduction: Current chemotherapy has limited effectiveness in hormone-refractory prostate cancer and new therapeutic strategies are urgently needed to improving the survival and quality of life in patients with hormone-refractory prostate cancer.

Current chemotherapeutic agents most commonly work by directly or indirectly inducing apoptosis, or programmed cell death in tumor cells. The impaired ability of prostate cancer cells to undergo apoptosis plays a key role in the resistance of prostate cancer cells to chemotherapy or radiation and for the failure of current treatment protocols for hormone-refractory prostate cancer. Hence, current and future efforts for designing new therapies to treat hormone-refractory prostate cancer must include strategies that specifically target resistance of prostate cancer cells to apoptosis.

Bcl-2 is a potent cellular inhibitor of apoptosis. Bcl-2 is overexpressed in 30-60% of prostate cancer at diagnosis but in nearly 100% of hormone-refractory prostate cancer. Prostate cancers that express high level of Bcl-2 are often resistant to chemotherapeutic agents or radiation therapy. Therefore, overexpression of Bcl-2 may play an important role to the high failure rate for current treatment of hormone-refractory prostate cancer. Hence, inhibition of the anti-apoptotic function of Bcl-2 represents a promising strategy for overcoming the resistance of prostate cancer to chemotherapy or radiation therapy and for developing an entirely new class of anticancer drugs for treatment of prostate cancer, especially hormone-refractory prostate cancer.

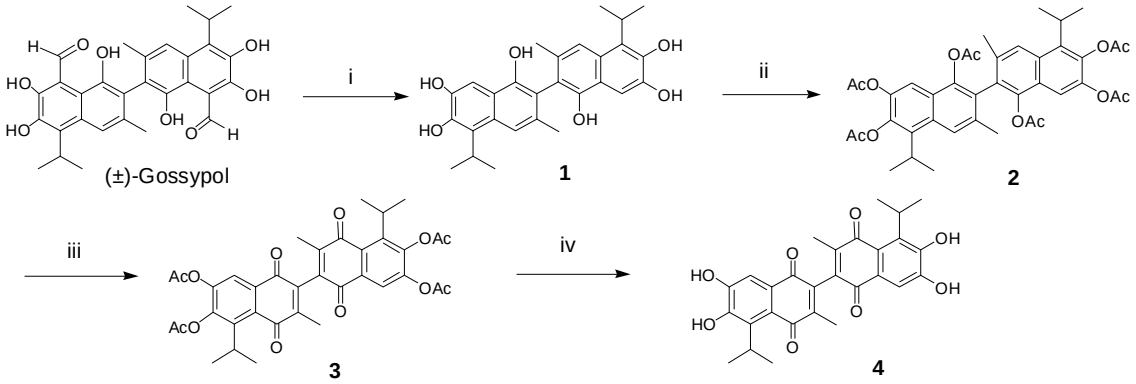
In this idea grant, we have proposed to test a potent and novel small-molecule inhibitor that we have discovered and synthesized in our laboratory for its mechanism of action and therapeutic potential for the treatment of human prostate cancer.

Body of the report:

Task 1. We will synthesize apogossypolone, (-)-apogossypolone and (+)-apogossyplone for *in vitro* and *in vivo* studies (1-6 months)

The synthesis of apogossypolone was very straightforward and was outlined in **Scheme I**. Racemic gossypol was treated with 40 % sodium hydroxide at 85 °C for 2 hours to remove the two aldehyde groups in gossypol, followed by acetylation using acetic anhydride to afford the (±)-hexaacetylapogossypol **2**. The yield for the two steps was 82 %. Compound **2** was transferred to (±)-tetraacetylapogossypolone **3** by oxidation using periodic acid. Deprotection of **3** in the presence of potassium carbonate in dioxane afforded (±)-apogossypolone **4** in very high yield (98%).

Scheme I. Synthesis of apogossypolone.



Reagents and conditions: (i) 40 % NaOH, 85 °C, 2 h; (ii) Ac_2O, iPr_2NEt, CH_2Cl_2, room temperature, 12 h, 82 % for two steps; (iii) Periodic acid, dioxane, 95 °C, 15 min, 55 %; (iv) K_2CO_3, dioxane, 70 °C, 5 h, then 4 M HCl, 98 %

Our attempt to prepare two enantiomers of apogossypolone was unsuccessful. Staring from each (-)-gossypol or (+)-gossypol enantiomer, hexaacetylapogossypol **2** was obtained in enantiomerically pure form. However, both (+)-**2** and (-)-**2** gave tetraacetylapogossypolone **3** in racemic form. This strongly suggests that the apogossypolone enantiomers racemize quickly at room temperature. This is in agreement with a previous report that gossypolone enantiomers racemize quickly at room temperature. Therefore, it was concluded that stable enantiomers of apogossypolone cannot be obtained at room temperature and the racemic apogossypolone is the only stable form of apogossypolone at room temperature.

Using the synthetic scheme outlined in Scheme I, we have now synthesized over 20 grams of apogossypolone. This amount is sufficient for our proposed in vitro and in vivo experiments as outlined in this proposal.

Task 2. We will evaluate their binding affinities to Bcl-2 and Bcl-xL proteins using our fluorescence polarization-based binding assays (3-6 months)

We have developed optimized sensitive, quantitative and competitive fluorescence-polarization (FP)-based binding assays for Bcl-2 and Bcl-xL using recombinant Bcl-2 and Bcl-xL proteins and fluorescently labeled Bid BH3 peptide. Using these assays, we have determined the binding affinities of apogossypolone to Bcl-2 and Bcl-xL proteins (Figure 1). As can be seen, apogossypolone binds to Bcl-2 with a K_i value of 170 nM and to Bcl-xL protein with a K_i value of 660 nM.

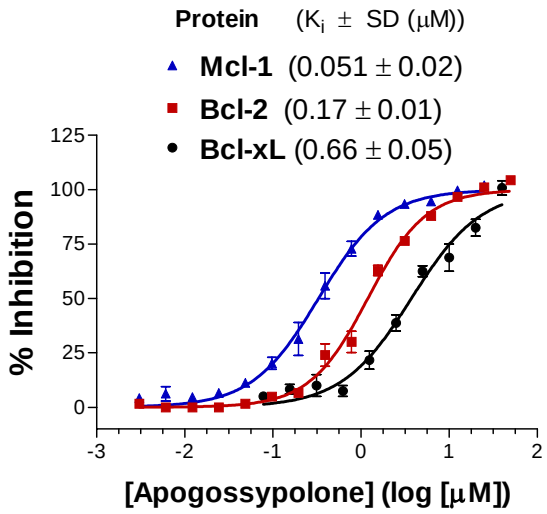


Figure 1 . Competitive binding curves of small-molecule inhibitor Apogossypolone to antiapoptotic members of Bcl-2 family of proteins (Bcl-2, Mcl-1 and Bcl-xL) as determined using a fluorescence-polarization based binding assay.

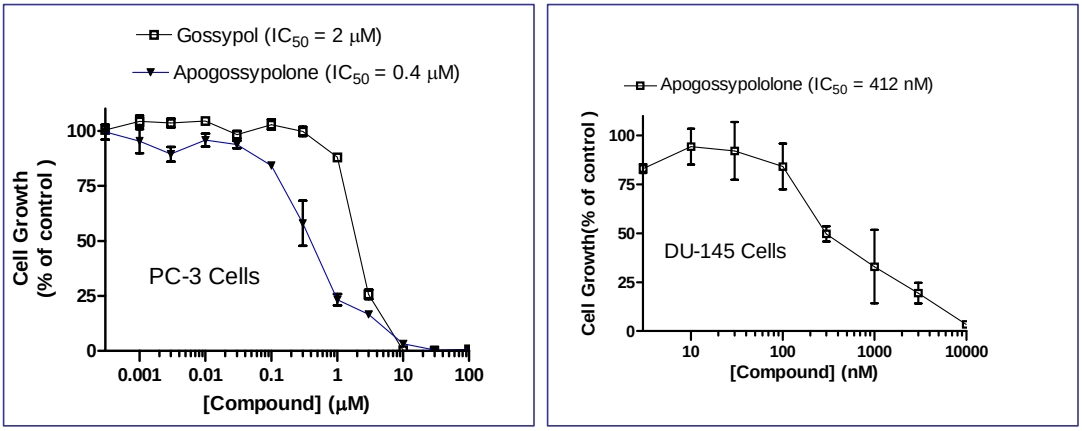
Recently, Mcl-1, a homolog of Bcl-2 protein, has emerged as an important molecular target for the design of new anticancer therapy. Like Bcl-2, Mcl-1 also potently binds to multiple pro-apoptotic members such Bim, Puma and Noxa and potently inhibits apoptosis. Although the role of Mcl-1 in human prostate cancer is not entirely clear, it has recently been shown that small-molecule inhibitors that only bind to

Bcl-2 and Bcl-xL proteins may have very limited activity due to overexpression of Mcl-1 in cancer cells. Therefore, we have developed a fluorescence-polarization-based assay for Mcl-1 using recombinant Mcl-1 protein and also fluorescence-labeled Bid BH3 peptide. We have determined the binding affinity of apogossypolone to Mcl-1 and the results are shown in Figure 1. As can be seen, apogossypolone binds to Mcl-1 with a K_i value of 51 nM. Thus, our binding data showed that apogossypolone binds to Bcl-2, Bcl-xL and Mcl-1 with high affinities and has the highest affinity to Mcl-1 protein. Our data thus suggests that apogossypolone may target all these three anti-apoptotic Bcl-2 members in cells and may have advantages as compared to small-molecule inhibitors that only target Bcl-2 and Bcl-xL proteins.

Task 3. We will test them in prostate cancer cells and normal cells with well-characterized Bcl-2 and Bcl-xL status for their activity and selectivity using MTT and apoptosis assays (4-12 months).

We have evaluated apogossypolone for its activity in inhibition of cell growth in androgen-independent PC-3 and DU-145 prostate cancer cell lines using the WST-based MTT assay. The results are shown in Figure 2. PC-3 and Du-145 have high levels of Bcl-2 and Bcl-xL proteins.

Figure 2. Inhibition of cell growth by apogossypolone in PC-3 and DU-145 androgen-independent human prostate cancer cell lines and comparison to that by gossypol.



As can be seen, apogossypolone is very potent in inhibition of cell growth in both PC-3 and DU-145 cell lines. The IC_{50} values are 400 nM and 412 nM, respectively, in

PC-3 and DU-145 cell lines. In direct comparison, gossypol has an IC_{50} value of 2 μM in PC-3 cell line. Therefore, apogossypolone is 5-times more potent than gossypol in inhibition of cell growth in PC-3 cell line.

Key Research Accomplishments:

(1). We have developed a simple and highly efficient synthetic method to synthesize large amount of apopgossypolone for the proposed in vivo studies to be carried out in year 2 of this grant. To date, we have synthesized over 20 grams of apopgossypolone. It was also discovered that the enantiomers of apopgossypolone quickly convert to each other. This significantly simplifies the development process for apopgossypolone as a potential drug candidate.

(2). We have now determined that apopgossypolone not only binds to Bcl-2 and Bcl-xL proteins with high affinities but also another important anti-apoptotic protein Mcl-1 with a high affinity. Thus, apopgossypolone is a potent, non-peptide small-molecule inhibitor of Bcl-2/Bcl-xL/Mcl-1. Such inhibitors will be much more effective in inhibition of cell growth or induction of cell death in cancer cells with overexpression of not only Bcl-2/Bcl-xL but also Mcl-1 than small-molecule inhibitors that only target Bcl-2 or Bcl-xL or Bcl-2/Bcl-xL.

(3). We have further demonstrated that apopgossypolone is effective and potent in inhibition of cell growth in androgen-independent PC-3 and DU-145 prostate cancer cell lines, 5-times more potent than gossypol.

(4). Our work in year 1 laid the solid foundation for the proposed in vitro and in vivo studies to be performed in year 2 and 3.

Reportable Outcomes:

(1). A manuscript described the design, synthesis and initial evaluation of apogossypolone as potent small-molecule inhibitors of Bcl-2/Bcl-xL/Mcl-1 is currently being prepared and will be submitted shortly.

(2). A patent application has been filed on the discovery of apogoysspolone.

Conclusions: Targeting the anti-apoptotic Bcl-2 members using non-peptide, small-molecule inhibitors is a new and exciting therapeutic strategy. Our work has led to the discovery of potent, non-peptide small-molecule inhibitor apogossypolone that not only binds to Bcl-2 and Bcl-xL proteins but also Mcl-1. Consistent with its strong binding affinity to Bcl-2 members, apogossypolone potently and effectively inhibits cancer cell growth in androgen-independent human prostate cancer PC-3 and DU-145 cell lines. Extensive in vitro and in vivo studies are being carried out to further test its therapeutic potential for the treatment of advanced, androgen-independent human prostate cancer.